Identification of a Pheromone Blend Attractive to *Manduca sexta* (L.) Males in a Wind Tunnel

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Analyses of solvent rinses of the external surfaces of pheromone glands excised from calling female tobacco hornworm moths, *Manduca sexta* (L.), revealed the presence of the following compounds: (Z)-9-hexadecenal, (Z)-11-hexadecenal, (E)-11-hexadecenal, hexadecanal, (E,Z)-10,12-hexadecadienal, (E,E)-10,12-hexadecadienal, (E,E,Z)-10,12,14-hexadecatrienal, (E,E,E)-10,12,14-hexadecatrienal, (Z)-11-octadecenal, (Z)-13-octadecenal, octadecanal, and (Z,Z)-11,13-octadecadienal. The two trienals were identified by mass and PMR spectral analyses and by ozonolyses, and their structures were confirmed by synthesis. In a wind tunnel male tobacco hornworm moths exhibit the same behaviors in response to a synthetic blend of all of the components, the gland rinse, or a calling female. Both (E,Z)-10,12-hexadecadienal and (E,E,Z)-10,12, 14-hexadecatrienal are required to stimulate males to complete the characteristic behavioral sequence: anemotaxis, approaching and touching the pheromone source, and bending their abdomens in apparent copulatory attempts. The other components of the blend may play more subtle roles.

Key words: insect behavior, aldehydes, 10,12,14-hexadecatrienal, tobacco hornworm, Sphingidae

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INTRODUCTION

The tobacco hornworm moth, *Manduca sexta* (L.), (Lepidoptera: Sphingidae) occurs over the greater part of the United States, the West Indies, Mexico, Central America, and parts of South America. It is a common pest of tobacco in the United States, and the larvae eat the leaves of a wide range of solanaceous plants including tomato, eggplant, jerusalem cherry, and potato [1].

More importantly, *M. sexta* has become the experimental insect of choice for many physiological and biochemical studies because it has a rapid rate of development and attains a weight of 8–9 g or more as a fully fed fifth instar larva [2]. Furthermore, it can be reared on artificial diet easily enough to make it readily available for a variety of research investigations. Thus, it has been used extensively to study olfactory neurophysiology [3–5], as well as biochemistry and metabolism [6,7] and endocrinology [8].

The search for the sex pheromone of *M. sexta* has involved many investigators over several years. Allen and Hodge [9] described the mating behavior and found the male to be strongly attracted to the female during about the third to the sixth hour of the scotophase. Subsequently, Allen et al. [10] reported the attractancy of diethyl ether extracts of female abdominal tips (the last two or three abdominal segments), obtained from females attractive to males during the most active period of the scotophase. Starratt et al. [11], with the aid of an electroantennogram bioassay, isolated and identified (*E,Z*)-10,12-hexadecadienal (E10,Z12-16:AL, bombykal) as an active component of the *M. sexta* pheromone. However, Starratt et al. [11] reported that there were indications for a second pheromone component, and we observed only a small percentage of males flying all the way to the source in response to E10,Z12-16:AL on filter paper in a wind tunnel (unpublished). On the other hand, we observed 70–80% of the males executing a complete sequence of mating behaviors including touching the source and bending their abdomens in apparent copulatory attempts in response to a hexane extract of the female pheromone gland [3].

We report here the isolation and identification of a second pheromonal component that is required, along with E10,Z12-16:AL, to elicit the complete sequence of behaviors in male *M. sexta* identical with those elicited by calling females and by rinses of female pheromone glands. Furthermore, we report the identification of a series of other 16-carbon and 18-carbon aldehydes from hexane rinses of pheromone glands. These other aldehydes may play a subtle role in the mating communication in this moth.

METHODS AND MATERIALS

Insect Rearing and Holding

M. sexta were reared at the Tobacco Research Laboratory, USDA, ARS (Oxford, NC) by the method of Baumhover et al. [12] and shipped as pupae to Gainesville, Florida. They were sexed in the pupal stage [13], and the sexes were held in cages in separate rooms at ca. 26°C under a reversed photoperiod, 14:10 LD. Enclosed adults were transferred to a separate cage every 24 h so that the age of the test insects could be determined. All adults were provided with a 5% sugar water solution on cotton.

Pheromone Extraction

Pheromone was obtained from calling 1–4-day-old virgin females. Females begin calling ca. 4 h after the beginning of the scotophase and may continue to call intermittently for 4–5 h. Females were considered to be calling when their ovipositor was obviously protruded. Calling females were removed from the holding cage, pressure was applied to the abdomen to cause the tip to protrude, and the pheromone gland, situated ventrolaterally in the intersegmental membrane between the eighth and ninth abdominal segments [14–16], was excised with small scissors. Care was taken to remove as little of the abdominal tip as possible with the gland, but it was impossible to avoid also taking some of the eighth and ninth segments. The gland was partially dipped (cut surface not submerged) in 100 μl of iso-octane or hexane contained in a 0.3 ml conical glass vial for 1 min. About 7–10 glands were rinsed by dipping in 100 μl of solvent, several batches of gland rinse were combined, and the solvent was then carefully evaporated by a fine stream of N_2 to a final concentration of 5 FGE*/μl and stored in a glass vial with a Teflon-lined screw cap at −60°C. For bioassay, the rinse obtained by dipping the glands was diluted to 2 FGE/0.25 ml with hexane. Diluted gland rinse for bioassay was stored in sealed ampules at −60°C.

Isolation and Identification

In initial attempts to isolate active components by micropreparative GLC a Varian (Sunnyvale, CA) model 1400 gas chromatograph equipped with a flame ionization detector, a 95:5 effluent splitter, and an external, dry ice-acetone-cooled fraction collector [17] was used. The gland rinse was chromatographed on a 4.4% OV101 on 120-140 mesh Chromosorb W glass column (2 m × 2 mm ID; Supelco, Bellefonte, PA), and fractions were collected in 1.0 mm (ID) × 280 mm glass capillary tubes. The injection port and detector temperatures were 200°C and 240°C, respectively, and the column was programmed from 80°C initial temperature to 230°C final temperature at 4°C/min. The carrier gas (He) flow rate through the column was 20 ml/min.

GLC analyses were conducted on Varian model 3700 and Hewlett-Packard (Palo Alto, CA) model 5890 gas chromatographs, both equipped with splitless capillary injector systems and flame ionization detectors. A Perkin Elmer (Norwalk, CT) Chromatographics 3 data system was used for data collection, storage, and subsequent analysis. Helium (linear flow velocity 19 cm/s) was used as a carrier gas. Fused silica capillary columns were used for analyses. Injections were made in the splitless mode at an initial column temperature of 60°C. After 30 s the injector was changed to the split mode and after 1 min TP of the column oven commenced. Other conditions for each column were 47 m × 0.25 mm (ID), OV101, TP at 10°C/min to 180°C; 50 m × 0.25 mm (ID), CPS-2 (Quadrex Corporation, New Haven, CT), TP at 30°C/min to 170°C; 25 m × 0.25 mm (ID) Carbowax 20M (Supelco, Bellefonte, PA), TP at 30°C/min to 170°C.

A 250 × 4.6 mm (ID) stainless steel HPLC column packed with 5 μm Adsorbo-

*Abbreviations used: FGE = female gland equivalent; ID = inside diameter; MS = mass spectrometric; NMR = nuclear magnetic resonance; PMR = proton magnetic resonance; TP = temperature programming.

sphere C18 (AllTech Associates, Deerfield, IL) was used for the analysis of the gland rinses and the purification of the naturally occurring conjugated diene and triene 16-carbon aldehydes. The mobile phase, 80/20 MeOH/H$_2$O, was pumped through the column at a flow rate of 2 ml/min by a Constametric IIG pump (Laboratory Data Control, Riviera Beach, FL) and the eluting components were detected with a Spectroflow 757 variable wavelength ultraviolet absorbance detector (Kratos, Ramsey, NJ). Preliminary experiments determined that the optimum wavelength for the detection of the trienals was 267 nm, while that for the dienals was 230 nm. Thus, in subsequent analyses the detector was set at 267 nm until the trienals eluted and then reset to 230 nm for the remainder of the run. Analyses were also conducted in which each of the optimum wavelengths was used for an entire analysis. A similar system was used for the purification of synthesized dienals and trienals but the column used was 250 × 22.5 mm (ID) packed with Adsorbosphere HSC18 (7 μm) and the MeOH/H$_2$O (87/13) mobile phase was delivered at a flow rate of 9 ml/min by a Laboratory Data Control Constametric II pump.

GLC-MS analyses were conducted with a Nermag (Houston, TX) model R1010 mass spectrometer in the chemical ionization mode. Samples were analyzed with methane and isobutane as reagent gases. The mass spectrometer was interfaced to a Hewlett-Packard model 5790 gas chromatograph equipped with a split/splitless injector and with a SGE OCI-3 cold on-column injection system. Natural and synthetic samples were analyzed with both injection systems. Helium was used as the carrier gas in all columns. In the split/splitless mode injections were made with a 30 s splitless delay. A 50 m × 0.32 mm (ID) BP1 (Scientific Glass Engineering, Inc., Austin, TX) fused silica column was operated at 80°C for the first 2 min and then TP at 32°C/min to 230°C. A 50 m × 0.25 mm (ID) CPA-1 (Quadrex Corporation) fused silica column was operated at 50°C for 2 min and then TP at 10°C/min to 180°C. In the on-column injection mode the injector was maintained at room temperature during a 20 s injection onto a 50 m × 0.25 mm (ID) OV1 fused silica capillary column. The column was held at 60°C for 1 min after injection and then TP at 15°C/min to 170°C.

PMR analyses were performed with a Nicolet 300 MHz Fourier transform NMR spectrometer interfaced to a Nicolet model 1280 data system. The natural trienals were purified by HPLC followed by micropreparative GLC on a 2 m × 2 mm (ID) glass column packed with 8.5% OV101 on 120/140 mesh Chromosorb W. The column was operated at 190°C isothermally in the Varian Model 1400 gas chromatograph. The compounds were collected in a glass capillary tube that had been previously cleaned by rinsing with water, acetone, and hexane, and then rinsed with hexane which was analyzed on the 47 m OV101 capillary to determine that no impurities were present. The collected material was transferred from the glass capillary, with benzene-D6, into an NMR tube, the top of which was 5 mm (OD), with a 50 × 2 mm (OD) coaxial extension at the bottom (Wilmad Glass Co., Buena, NJ). Data points (16 K) were collected with a 6 μs pulse.

The trienals were collected for ozonolysis in the same manner as for NMR, but were rinsed from the collection tube into a 100 μl conical microvial with hexane. Micro-ozonolysis of the GLC-purified trienals was conducted by the method of Beroza and Bierl [18,19]. Ozone was introduced into a hexane solu-

tion of the compound at −78°C. After saturation of the solution with ozone reductive cleavage of the ozonide was accomplished with triphenyl phosphine and the products were analyzed by capillary GLC on OV101.

Most of the saturated and monoene synthetic standards used in this study were obtained from commercial sources and purified by HPLC on a 25 × 2.5 cm (OD) AgNO$_3$-coated silica column eluted with toluene [20]. (Z)-11-Octadecenal (Z11-18:AL) and (Z)-13-octadecenal (Z13-18:AL) were synthesized in this laboratory by standard methods [21–23] and purified by AgNO$_3$-HPLC. Both (E,Z)- and (E,E)-10,12-hexadecadienal and all four isomers of 11,13-octadecadienal were synthesized in this laboratory by the general method of conjugated diene synthesis described by Zweifel and Backlund [24] and applied to the synthesis or functionalized conjugated dienes by Doolittle and Solomon [25]. These compounds were purified by reverse phase HPLC as described above. All synthesized compounds were analyzed on both polar and nonpolar capillary GLC columns and determined to be greater than 99% pure.

Wind Tunnel Bioassays

Bioassays were conducted in a wind tunnel 1 m wide × 1 m high × 2.9 m long. A fan forced room air at ca. 26°C through the tunnel at ca. 0.3 m/s and the air was exhausted from the wind tunnel room. The wind-tunnel room was maintained under a reversed photoperiod of 14:10 LD. The light intensity during the scotophase was about 1.5 lux. Bioassays were conducted during the second to fifth hours of the scotophase.

Males were held in the wind-tunnel room and used for bioassay at 1–4 days after eclosion. Females were brought into the wind-tunnel room only when they were to be used in the bioassays.

To test the responses of a male to a live virgin female, a calling female was placed at the upwind end of the wind tunnel on a piece of screening (curved so it would stand freely) ca. 36 cm above the tunnel floor to ensure that her pheromone would be carried in the air stream of the tunnel. In some cases the wings of the female were partially clipped prior to the calling period (this did not prevent calling) to ensure that she would not fly away during the test period.

Ampules containing various concentrations of gland rinse or of synthetic blends in 0.25 ml of solvent were opened in a fume hood in the wind-tunnel room and poured onto a 7 cm (diameter) piece of brown construction paper folded into a triangular shape. The solvent was allowed to evaporate in the hood, and then the paper triangle was suspended in the wind tunnel 36 cm above the floor and 24 cm from the upwind end. The location and dimensions of the pheromone plume were determined by releasing titanium tetrachloride smoke from the same point in the wind tunnel.

Test insects were released into the wind tunnel at the center of the downwind end from an 8.3 cm diameter × 16 cm long paper carton fitted with a screen on the downwind end to allow air to flow through the carton. When the insect flew from the carton, the test was begun and the test insect was observed for a maximum of 5 min in bioassays during the isolation and identification stages of this study. In subsequent experiments, in which the responses of males to various synthetic blends, the gland rinse, and a range of doses of

certain blends were analyzed, the test was terminated after 2 min. Males (ca. 10%) that were determined to be incapable of flight during the selection process were not tested.

The responses of M. sexta males to calling female M. sexta and to the gland rinse in the wind tunnel were similar to the precourtship behavior of male Heliothis virescens (F.) described by Teal et al [26]. The following behaviors were observed and recorded for each insect tested in the wind tunnel: random flight, upwind taxis in the plume toward the pheromone source (taxis), approach to within 10–30 cm of the source (approach), diminished rate of forward advance or hovering with 10 cm of the source (hover), making contact with the source (hit), and bending the abdomen (bend) in apparent copulation attempts with the females or paper. Behavioral durations and transitions were recorded with an Epson HX-20 (Torrance, CA) portable computer.

No more than five males were flown to each sample or to each dose in dose-response tests during one day. Each male flown was considered one replicate. Statistical analyses were performed with a chi square test of independence and Yates correction at a 0.05 probability level.

RESULTS AND DISCUSSION

Initially the last two to three segments on the tip of the abdomen were clipped from females that were observed to be calling during the scotophase as described previously [10]. However, diethyl ether or hexane extracts of the abdominal tips contained large amounts of lipids and it was difficult to separate the active pheromone from the crude extract.

Location of the pheromone gland [14] made it possible to excise this structure without obtaining excessive amounts of other tissue or material from the abdomen. Dipping just the uncut surface of excised glands in solvent yielded a very clean extract (rinse) as determined by capillary GLC (see later). When extracts prepared in this manner were bioassayed in the wind tunnel males exhibited the same behavior as they did in response to calling virgin females. Of 409 males tested, 85.6% proceeded through the sequence of behaviors including taxis, approach, hover, and touching (hit) the paper containing 2 FGE of gland rinse, while 79.5% (N = 39) exhibited these same behaviors in response to calling virgin females [3]. Typically the gland rinse was active over a wide range of doses from 0.002 to 2 FGE.

Initial attempts to fractionate the gland rinse by micropreparative GLC on the packed OV101 column resulted in loss of activity as determined by the wind tunnel bioassay. Activity could not be recovered by recombining GLC fractions. Occasionally activity could be recovered in the fraction expected to contain 16-carbon aldehydes when crude gland extracts were injected on the packed column. However, this could not be repeated reliably and rechromatography of these active fractions or of active fractions from HPLC on the packed GLC column nearly always resulted in loss of activity. Activity could not be detected even when GLC fractions were bioassayed over a range of doses from 6 to 0.2 FGE.

When the gland rinses were analyzed by capillary GLC on both polar and apolar columns several peaks were revealed. Comparison of the retention times

on all three capillary columns and the mass spectra of the components of the gland rinse with those of authentic standards permitted the identification of the compounds listed in Table 1, with the exception of the trienals. The quantity of each compound found in 1 FGE was determined by comparison of peak areas with those of the internal standards, tridecanyl acetate (S-13:Ac) and pentadecanyl acetate (S-15:Ac.), added in known quantities to the samples to be analyzed. Figure 1B shows the analysis of the gland rinse on an OV101 column.

A synthetic blend containing all of the compounds identified thus far, in the same ratio found in the gland rinse, was prepared. GLC analyses on the OV101 (Fig. 1A) and the polar capillary columns indicated that the ratio and retention times of the compounds were nearly identical with those of the identified compounds in the gland rinse. This synthetic blend was bioassayed in the wind tunnel over a range of doses from 0.008 to 2.8 FGE. However, the synthetic blend elicited very little activity compared to the gland rinse.

Since preparative GLC appeared to destroy the pheromonal activity of the gland rinse, HPLC was employed to isolate and purify the active components. Reverse phase HPLC of the gland rinse with the detector wavelength set at 267 nm and at 230 nm indicated that there were four detectable peaks with the optimum wavelength for the first two being about 267 nm and for the last two about 230 nm (Fig. 2A). Absorption at these two wavelengths suggested that the first two peaks might contain conjugated triolefinic systems and the latter two conjugated diolefinic systems [27,28]. When these peaks were collected individually, recombined, and bioassayed at a concentration of 1 FGE, the recombination was as attractive to males in the wind tunnel as the gland rinse. Only samples that contained both peak 1 and peak 3 were active in the bioassay, and a combination of peaks 1 and 3 was equal to the recombination of peaks 1–4 and to the gland rinse, at concentrations of 0.1 to 1 FGE, in eliciting the full range of responses by males in the wind tunnel: percent hit at 0.3

TABLE 1. Compounds Identified in Solvent Rinses of Female *Manduca sexta* Pheromone Glands

Components[a]	Peak No.[b]	ng/FGE, ±SD
Z9-16:AL	1	0.8 ± 0.4
Z11-16:AL	2[c]	13.4 ± 4.6[c]
E11-16:AL	2[c]	6.8 ± 2.3[c]
S-16:AL	3	15.7 ± 6.1
E10,Z12-16:AL	4	23.8 ± 6.2
E10,E12-16:AL	5	3.9 ± 0.9
E10,E12,Z14-16:AL	6	11.3[d]
E10,E12,E14-16:AL	7	1.2[d]
Z11-18:AL	8	6.2 ± 2.5
Z13-18:AL	9	2.2 ± 0.8
S-18:AL	10	4.8 ± 2.0
Z11,Z13-18:AL	11	1.4 ± 0.4

[a]A standard shorthand notation is used for pheromone molecules of this type. For example, (Z)-11-hexadecenal is abbreviated Z11-16:AL, (E,Z)-10,12-hexadecadienal is E10,Z12-16:AL.
[b]Peak numbers on chromatogram in Figure 1B.
[c]These two components were resolved and their relative quantities determined on the CPS-2 capillary GLC column.
[d]The trienals could not be accurately quantified by GLC because of their instability.
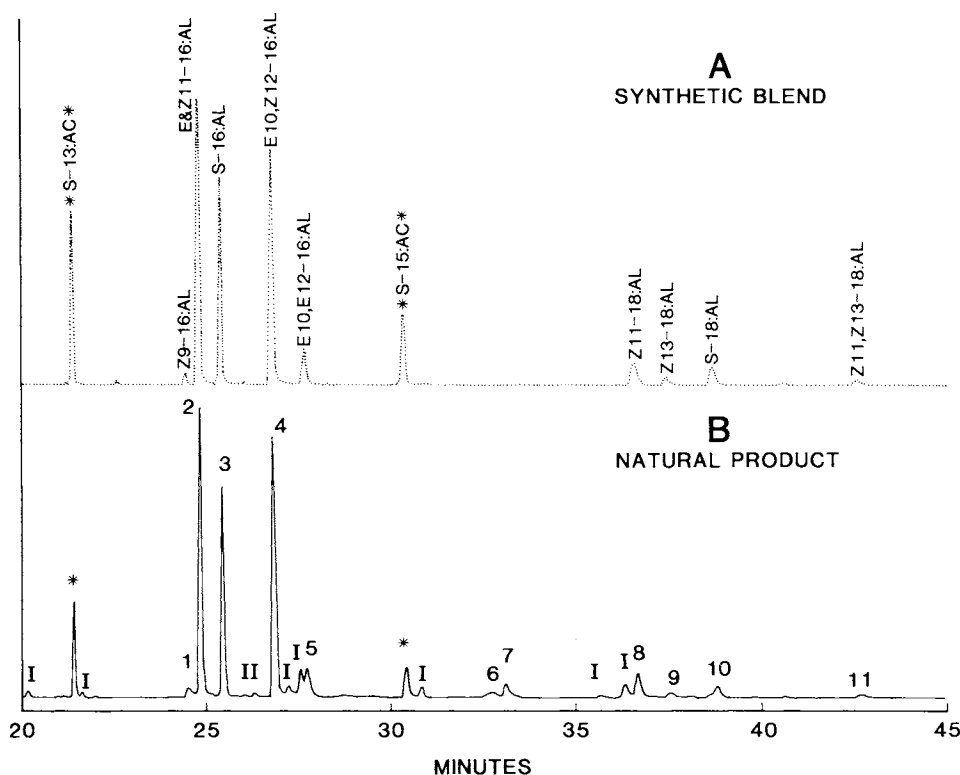
Fig. 1.   GLC analysis on the OV101 fused silica column of a synthetic blend (A) and 5 FGE of the gland rinse in hexane (B). Internal standards, tridecanyl acetate and pentadecanyl acetate, are indicated by asterisks. Abbreviations are the same as in Table 1. I = solvent impurities.

FGE concentration was 93 (N = 32) for peak 1 + 3, 88 (N = 30) for 1 + 2 + 3 + 4, 100 (N = 10) for gland rinse, 4 (N = 28) for solvent blank, 11 (N = 28) for peak 1 + all fractions preceding peak 1 and following peak 4, and 25 (N = 28) for peak 3 + all fractions preceding peak 1 and following peak 4.

Capillary GLC and GLC-MS analyses of these peaks revealed that peaks 3 and 4 were the previously identified (E,Z)- and (E,E)-10,12-hexadecadienals, respectively, by congruence of their retention times and mass spectra with those of authentic standards. Peaks 1 and 2 from the HPLC corresponded in capillary GLC retention time and mass spectra with two small peaks, 7 and 6, respectively (Fig. 1B), observed but not identified in the capillary gas chromatograms of the gland rinse. Capillary GLC also indicated that each of these four components was free of the saturated and monene aldehydes found in the gland rinse.

Isobutane ionization mass spectra of HPLC peaks 1 (Fig. 3B) and 2 indicated that they were 16-carbon aldehydes with three double bonds. Additionally, when the spectra were compared to that of E10,Z12-16:AL (Fig. 3A), certain similarities were noted. In particular, the ions at m/z 183 and 165 were found in all three spectra. In the spectrum of E10,Z12-16:AL, these ions are diagnostic for location between carbons 10 and 11 of the double bond nearest the
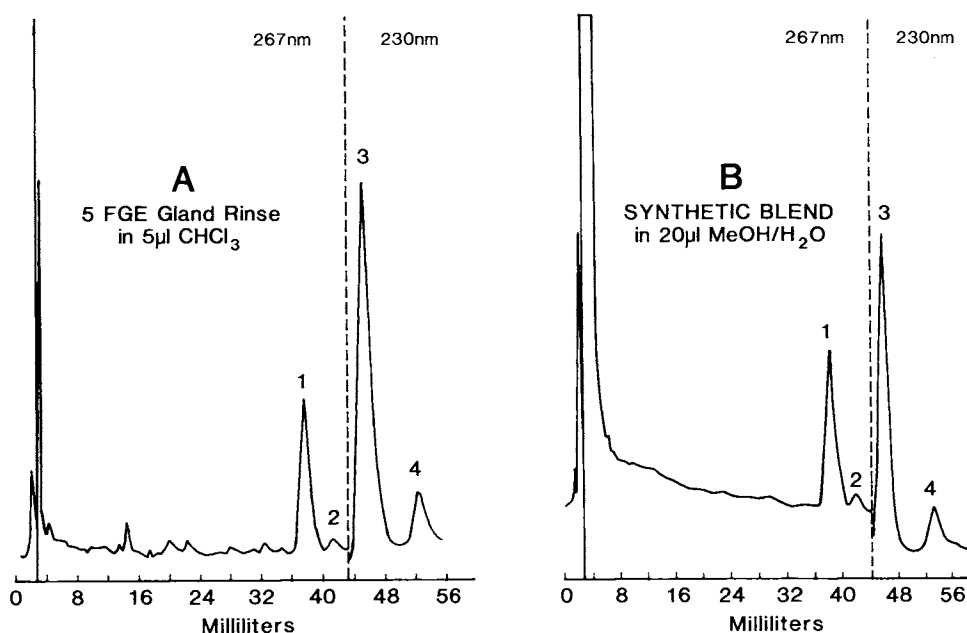
Fig. 2.   HPLC analysis on the 4.6 mm ID Absorbosphere C18 column of 5 FGE of the gland rinse (**A**) and of the synthetic blend (**B**) composed of E10,E12,Z14-16:AL, **peak 1**; E10,E12,E14-16:AL, **peak 2**; E10,Z12-16:AL (75ng), **peak 3**; and E10,E12-16:AL, **peak 4.** This is the same four-component synthetic blend tested in the wind tunnel, Figures 5 and 6.

functional group in the conjugated system [29,30]. In E10,Z12-16:AL there is also an ion at m/z 85 diagnostic for locating the last double bond in the conjugated system between carbons 12 and 13, thus leaving three carbons beyond the conjugated diene in the chain. In the mass spectra of the trienals there were no obvious ions in the low-mass end of the spectrum that could not be attributed to background. If a similar mechanism of fragmentation occurs in isobutane ionization mass spectroscopy in conjugated trienes as in conjugated dienes [31,32], then a methyl group at the end of the chain would result in an ion at m/z 57, which would be obscured by the $C_4H_9^+$ ion of the reagent gas. However, an ethyl group on the end of the chain is clearly recognizable by a diagnostic ion at m/z 71 that is detectable above the background ions at that mass [29,30]. Thus, the absence of an ion at m/z 71 in the spectra of the trienals indicated that a terminal ethyl group was not present.

Micro-ozonolysis of the trienals yielded a product identical in capillary GLC retention time with the 1,10-decandial produced by micro-ozonolysis of E10, Z12-16:AL, confirming the location at position 10 of the first double bond in the conjugated system.

The PMR spectrum (Fig. 4A) of the major natural trienal (peak 1, Fig. 2A) collected from GLC suggested the presence of a conjugated triene system by analogy with the PMR spectra of other compounds with conjugated triene systems [33]. The presence of four clusters of peaks in the olefinic region of the spectrum is very similar to that of methyl (Z,E,E)-9,11,13-octadecatrienoate. Integration of the spectrum indicated that more than four olefinic protons were
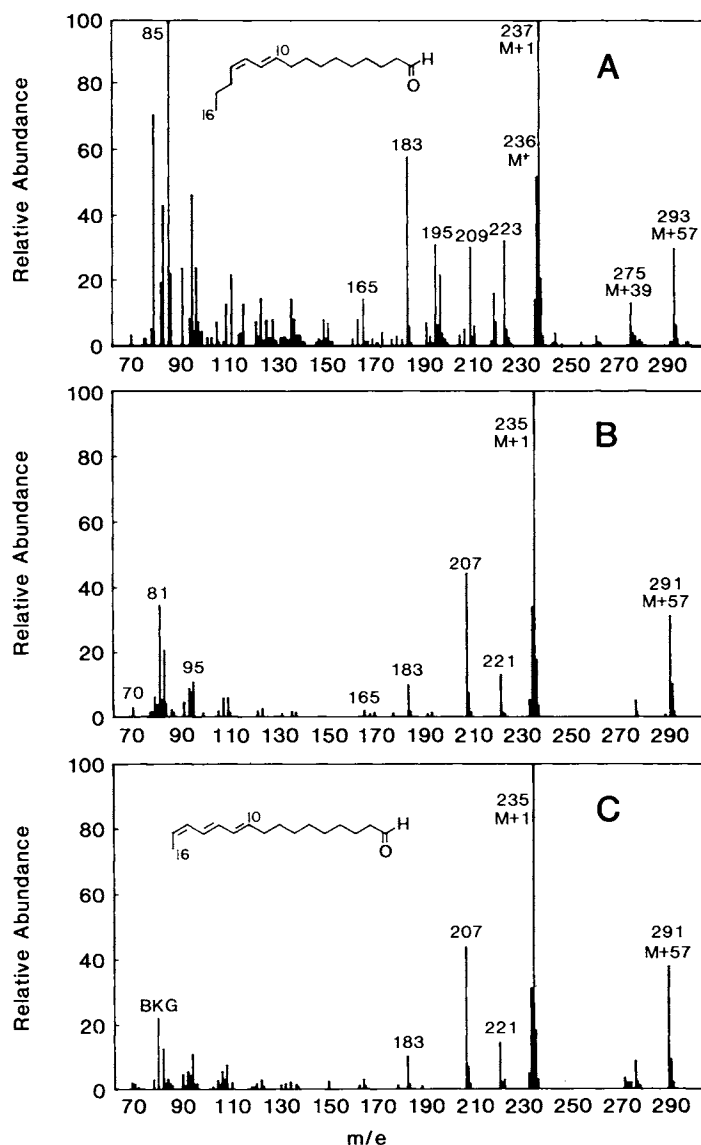
Fig. 3.   Isobutane ionization mass spectra of synthesized E10,Z12-16AL, (A), peak 1 from HPLC fractionation of the gland rinse (B), and synthesized E10,E12-Z14-16:AL (C).

present in the compound. This supported the possibility that the natural compound was a conjugated triene rather than an allene. Furthermore, the possibility of a terminal double bond was eliminated by the PMR spectrum. Although our original estimate before preparative GLC indicated that about 6 μg of the major trienal was available, after collection from GLC we estimated that we had less than 1 μg for NMR. Also, after the NMR spectrum was obtained, analysis of the contents of the NMR tube by capillary GLC indicated that both trienal peaks 1 and 2 were present in a ratio of about 80/20, respectively. Thus,
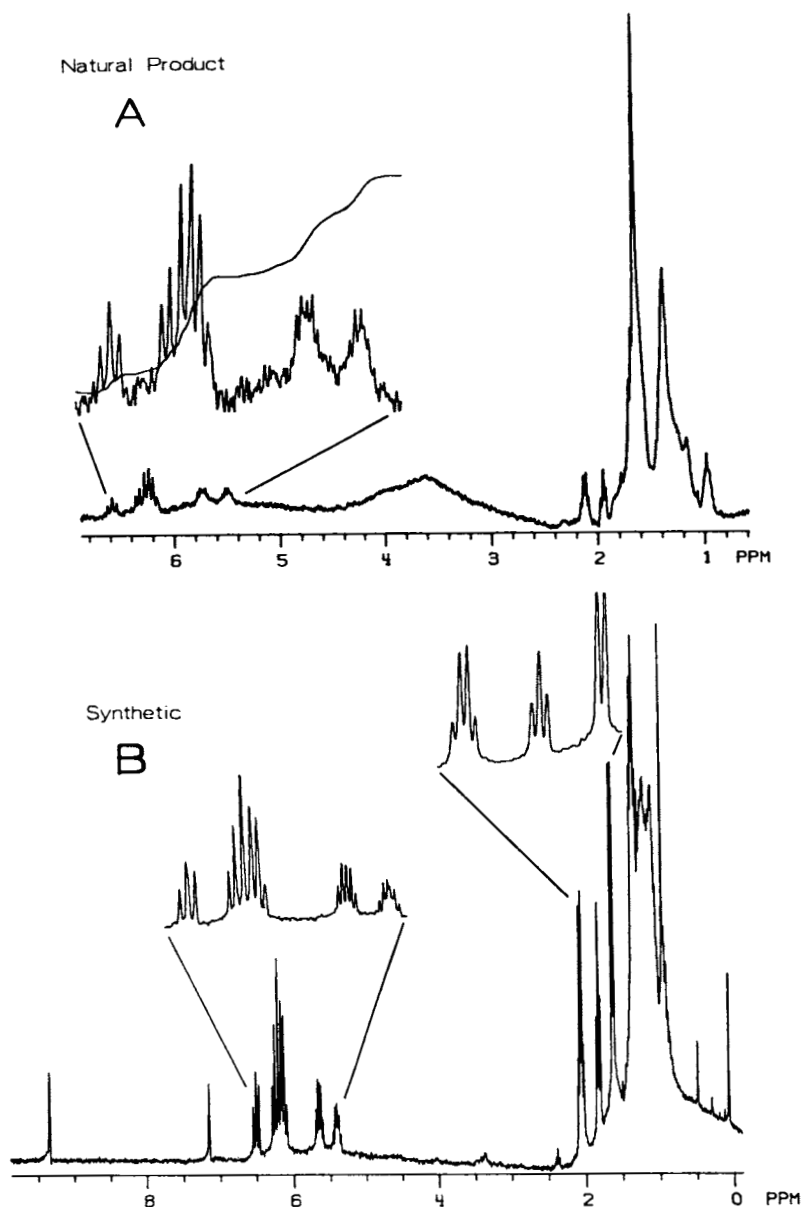
Fig. 4.   PMR spectra of the natural trienal collected from GLC (**A**) and of synthesized E10,E12, Z14-16:AL (**B**). Both samples were dissolved in benzene-D_6.

because of the instability of the trienals it was impossible to obtain a completely reliable spectrum.

The data obtained at this point supported the identification of the two peaks from HPLC as 16-carbon aldehydes with three olefinic bonds. The first two bonds in the chain were firmly established to be in positions 10 and 12 by isobutane ionization mass spectrometry and the location of the first bond was supported by ozonolysis. Both the UV absorbance at 267 nm and the NMR

spectrum strongly suggested a conjugated triene system. Therefore the eight isomers of 10,12,14-hexadecatrienal were considered as possibilities for the structures of the two trienals. Since the conjugated dienals produced by the insect are (E,Z)- and (E,E)-10,12-hexadecadienal, we considered that the most likely configurations for the bonds at positions 10 and 12 in the trienals were EZ and EE.

Three isomers of the conjugated trienal—E,Z,E, E,E,Z, and E,E,E—were synthesized. The full details of these syntheses will be published elsewhere [34].

Each synthesized isomer was purified by HPLC on the 22.5 mm (ID) reverse phase column and analyzed by HPLC on the 4.6 mm (ID) reverse phase column, by GLC on all three capillary columns, by GLC-MS, and by NMR. The (E,E,Z)-10,12,14-hexadecatrienal (E10,E12,Z14-16:AL) was identical with peak 1 from HPLC of the female gland rinse in HPLC and GLC retention times on all columns and in its mass spectrum (Fig. 3C). Furthermore, its PMR spectrum (Fig. 4B) was identical in the olefinic region with that of the natural trienal(s) collected from GLC. The (E,E,E)-isomer corresponded in retention times and mass spectra to peak 2 from HPLC of the gland rinse. The (E,Z,E)-isomer had different HPLC and GLC retention times from those of the two natural product trienals.

To verify the activity of synthetic E10,E12,Z14-16:AL and to evaluate the response of *M. sexta* males to blends of the synthetic compounds, a series of behavioral experiments was conducted in which the behaviors of males in a wind tunnel were observed and recorded. Initially the behaviors of males in response to a range of doses of each of four blends were recorded. In these experiments the blends tested consisted of the gland rinse, a synthetic blend containing all of the compounds identified in the gland rinse (total synthetic blend); a four-component blend of synthesized E10,E12,Z14-16:AL, E10,E12, E14-16:AL, E10,Z12-16:AL and E10,E12-16:AL (Fig. 2B); and a two-component blend of synthesized E10,E12,Z14-16:AL and E10,Z12-16:AL. Synthetic blends were prepared by adjusting the amount of the trienals in the blend to produce peaks of the same relative size to the dienals, when analyzed by HPLC (Fig. 2B), as were found in the gland rinse. It was not possible to obtain enough pure material of either trienal to weigh samples accurately, nor was it possible to determine accurately the amount of trienals in the gland rinse because of their instability. All the other compounds were formulated by weight, and the synthetic blend was analyzed by capillary GLC to verify that ratios were the same as those of the components in the gland rinse.

The results of the dose-response tests for the four blends are presented in Figure 5. It is evident from these data that at the higher concentrations the two-component blend is equivalent to the gland rinse in eliciting the complete sequence of male behaviors. At concentrations of less than 0.02 FGE it appears that the two- and four-component blends are less effective than equivalent amounts of the gland rinse in eliciting the full range of responses. However, blends were not directly compared at concentrations of less than 0.02 FGE.

Additionally, because electrophysiological studies [35,36] indicated that receptors in the antennae and olfactory neurons in the antennal lobes of *M. sexta* males responded to the EEE-trienal (see later), two blends of E10,Z12-16:AL and E10,E12,E14-16:AL were prepared. In one of these blends the ratio of the
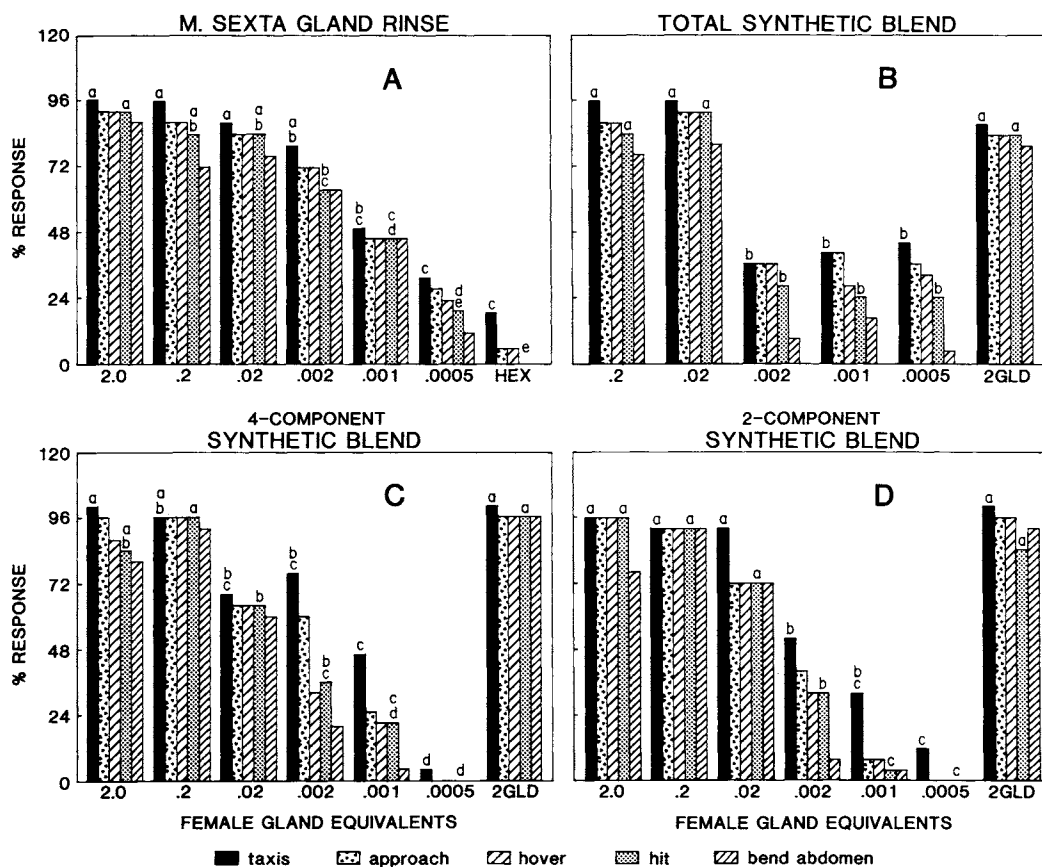
Fig. 5.   Behaviors exhibited by male tobacco hornworm moths in a wind tunnel to a range of doses of A) the female pheromone gland rinse; B) a synthetic blend of all the compounds identified in the gland rinse; C) a blend of synthesized E10,E12,Z14-16:AL, E10,E12,E14-16:AL, E10,Z12-16:AL and E10,E12-16:AL in the same ratio they occur in the gland rinse as determined by HPLC (Fig. 2B); and D) a blend of synthesized E10,E12,Z14-16:AL and E10,Z12-16:AL in the same ratio found in the gland rinse by HPLC. N = 25 for all treatments. The results of statistical analyses of the data are indicated by letters on the graph only for the behaviors taxis and hit. For each behavior in an experiment bars not topped by the same letters differ significantly at the 0.05 probability level as determined by chi square test with Yates' correction.

two compounds was identical with that found in the gland rinse. In the other blend the proportion of the E10,E12,E14-16:AL was increased to equal that of the EEZ-trienal in the gland rinse. In the wind tunnel the response of males to each of these two blends, to the two-component blend of E10,Z12-16:AL plus E10,E12,Z14-16:AL, and to E10,Z12-16:AL alone was compared at a concentration of 0.02 FGE. The response of males to the blends containing the EEE-trienal was no different than to E10,Z12-16:AL alone.

Finally, an experiment comparing several blends at a concentration of 0.02 FGE was conducted. The results (Fig. 6) clearly indicate that both E10,Z12-16:AL and E10,E12,Z14-16:AL are required to elicit the full sequence of behaviors exhibited by male *M. sexta* in response to the natural pheromone blend. Some
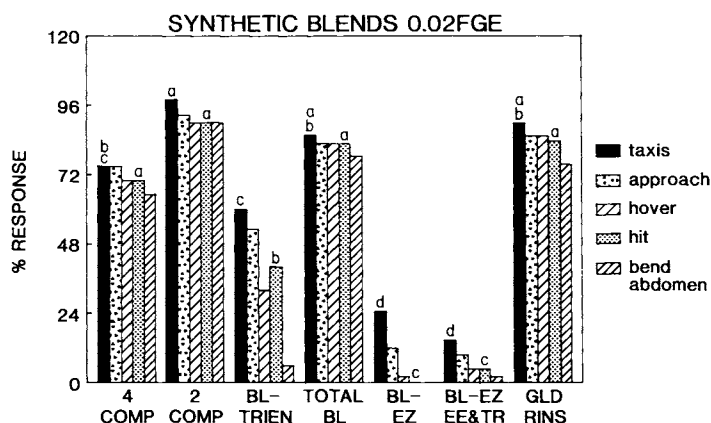
Fig. 6. Comparison of the behaviors exhibited by male tobacco hornworm moths in a wind tunnel to 0.02 FGE doses of the following blends: **4-comp,** synthesized E10,E12,Z14-16:AL, E10,E12,E14-16:AL, E10,Z12-16:AL, and E10,E12-16:AL; **2-comp,** E10,E12,Z14-16:AL and E10,Z12-16:AL; **Total BL,** all components listed in Table 1; **BL-TRIEN,** all components in Table 1 except the two trienals; **BL-EZ,** all components in Table 1 except E10,Z12-16:AL; **BL-EZ,EE, and TR,** all components in Table 1 except E10,Z12-16:AL, E10,E12-16:AL, and the two trienals; **GLD RINS,** gland rinse at 2.0 FGE dose. N = 40 for all treatments. For each of the two behaviors, taxis and hit, bars not topped by the same letters differ significantly at the 0.05 probability level (chi square test with Yates' correction).

of the other components in the blend could possibly play more subtle, as yet undefined roles in this communication system.

Clarification of the roles of the various components of this pheromone system will require further behavioral analyses with blends formulated to release precise ratios of components at controlled rates. It is very likely that the release rates and/or ratios of the components from the paper dispenser used in this study differed with different blends. However, despite the lack of precision in these formulations there are several points worth noting. With the total synthetic blend (Fig. 5B) there was a sharp decrease at the 0.002 FGE dose in the number of males entering taxis. Of those entering taxis, however, a high percentage approached and hit the target. Thus, it appears that the synthetic blend may lack a component or may not release the right ratio of components required for initiation of taxis when the concentration is low. It is interesting that these behaviors did not decrease signficantly with further decreases in the dose of this blend to 0.0005 FGE. The same decrease in taxis between 0.02 and 0.002 FGE was observed with the two-component blend (Fig. 5D), but in this case the responses to 0.001 and 0.0005 FGE continued to decrease. In contrast, with the four-component blend (Fig. 5C), the decrease in response occurred at the 0.02 FGE dose. Also, in Figure 6 it can be seen that in a direct comparison of blends at the 0.02 FGE dose, the four-component blend was significantly less effective than the two-component blend in initiating taxis. However, there was no statistical difference in the percentage of males that hit the source and attempted to mate in response to these two blends.

Electrophysiological studies have shown that E10,Z12-16:AL selectively stimulates one of the two pheromone receptor cells in each trichoid sensillum on

the antenna of *M. sexta* males. In most of these sensilla the second receptor responds to E10,E12,Z14-16:AL, but in some sensilla the second receptor responds to the EEE isomer [35]. Furthermore, olfactory (deutocerebral) interneurons in the antennal lobes of males exhibit a wide range of responses to pheromonal stimulation of the ipsilateral antenna. Christensen et al. [36] have shown that while all of the 16-carbon aldehydes found in solvent rinses of the *M. sexta* female's pheromone gland elicit some form of response in olfactory interneurons in males, E10,Z12-16:AL, E10,E12,Z14-16:AL, and E10,E12,E14-16:AL evoke the greatest responses. Those findings strongly suggest that all the 16-carbon aldehydes found in rinses of the pheromone gland have a role in the communication system of this insect. It is conceivable that the 18-carbon compounds are biosynthetic "by-products" that are not involved in mating communication. Of course, they may have a behaviorally antagonistic role in interspecific communication with other closely related species.

Although aliphatic conjugated triene systems are labile and relatively rare in nature, they have been found in a few plant species [28]. We are aware of only one other published report of a conjugated triene insect pheromone. The pheromone of the mulberry pyralid, *Glyphodes pyoalis*, was reported to be 10,12,14-hexadecatrienyl acetate, although behavioral studies with the synthesized pheromone were not reported and the geometry of the olefinic bonds was not established conclusively [37]. Several conjugated diene pheromones have been reported, including bombykol, E10,Z12-16:OH, the first insect pheromone to be isolated and identified [38]. Subsequent reports have indicated that the biosynthesis of bombykol in the silkworm moth proceeds through a precursor, (Z)-11-hexadecenoate, formed by the action of a ▲11-desaturase on hexadecanoate [39–41]. Because the *M. sexta* pheromone glands produce both S-16:A1 and Z11-16:A1 in relatively large amounts, it is tempting to speculate that the dienals and trienals are likewise produced by further desaturation and isomerization of the (Z)-11-hexadecenoate in this species.

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